TYPE 1/TYPE 2 CYTOKINE SERUM LEVELS AND ROLE OF INTERLEUKIN-18 IN CHILDREN WITH STEROID SENSITIVE NEPHROTIC SYNDROME

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Abstract:
Aims: In view of the conflicting evidence of helper T cell type 1 (Th1) or type 2 (Th2) pattern of cytokine synthesis in steroid sensitive nephrotic syndrome (SSNS) this study aims to assess type-1 /type-2 cytokines in different stages of SSNS and define the potent involvement of IL- 18
Methodology: We prospectively studied thirty children with steroid sensitive nephrotic syndrome (SSNS), aged 2–12 years. Thirty children were in active stage before treatment initiation, the same 30 children in remission still on steroids; 21/30 in remission off steroids as well. The control group Included 30 healthy age and sex matched siblings. Using ELISA technique we assessed serum levels of Serum, IL-2, IFN-γ, IL-4, IL-13 and IL-18 in different stages of the disease and in controls.
Results: IL-2 levels were not significantly different in children of all disease stages of SSNS and controls ($p > 0.05$). Levels of IL-4, IL-13 and IL-18 were significantly higher in the active stage of SSNS compared with the remission stages and controls ($p<0.05$). But, serum IFN-γ was significantly lower in children with active disease compared with remission stages and controls ($p<0.05$). In children with SSNS, of all disease stages, serum levels of IL-18 were significantly correlated with both IL-4 and IL-13 ($r = 0.72$ and $p<0.0001$, $r = 0.82$ and $p<0.0001$, respectively).
Conclusion: Children with active SSNS seem to have a shift to type-2 cytokine production, and IL-18 expression is significantly correlated with this type-2 immune response.

Key Words: Type 1 cytokine, Type 2 cytokine, Nephrotic Syndrome

Introduction:

Idiopathic nephrotic syndrome (INS) is considered to be an immune-mediated disease [1]. But, how T-cells affect the course of the disease remain unanswered question. However, circulating factors were proposed to be released from activated T-cells which may affect the pathogenesis of the disease. Several cytokines are considered prime candidates for the role of mediators of INS [2].
Based on the predominant cytokines, the immune response can be functionally subdivided into type-1 and type-2. Type-1 response, which normally prevails, and produce interferon-gamma (IFN-γ) and interleukin (IL)-2 and enhance both the production of complement-fixing and opsonizing antibodies and, macrophage activation resulting into delayed type hypersensitivity (DTH), Whereas type-2 response is dominated by IL-4 and IL-13 and provide optimal help for antibody production; and promote both mast cell growth and eosinophil differentiation and activation, resulting in humoral responses [3].

Measurement of cytokine level in serum or urine in INS patients have been performed by many investigators. Increased levels in serum or urine in relapse were reported in interleukin (IL)-2 [4], soluble IL-2 receptor [5-8], and (IFN-γ) [5, 9], IL-4 [9, 10], IL-12 [11],
INS has been suggested to be a Th2-dependent glomerular disease [12] depending on the evidenced association between it and atopy and allergy [13-15], many allergic disorders, as asthma, allergic rhinitis and eczema, are typically linked to the presence of a Th2 immunologic response.

Further support to the hypothesis that INS is a Th2-dependent glomerular disease comes from the elevated serum IgE and preservation of IgG4 observed in INS, [16-18]. In addition, levels of IL-4 and IL-13, which are two of the major Th2- associated cytokines, have been shown to be elevated in INS patients in relapse [9, 10, 16, 19]. . However, contradictory observations have been also reported, [19- 21].

IL-18 is an exclusive cytokine which, can stimulate both type-1 and type-2 immune responses depending on the cytokine enviroment [4].

Since Matsumoto and Kanmatsuse [22] elegantly demonstrated that IL-18 was involved in the active stage of the disease, and that there was a positive correlation between IL-18 production and disease activity was by, efforts have been made to identify the pathogenetic vascular permeability factors VPF(s) released from T-cells, such as cytokines, as well as to clarify the participated cells in its pathogenesis.

For the last two decades many studies investigated the cytokine pattern in SSNS with sometimes conflicting results [5–7]. So, it is still questionable is type-2 response dominated in active SSNS.

Besides, literature is lacking regard the involvement of IL-18.

The current study aims to evaluate prospectively type-1/type-2 cytokine profile in the same children with different stages of primary SSNS and to investigate the potent involvement of IL-18.

Subjects and methods:

This is a cross-sectional prospective longitudinal study; the study was carried on in Madinah Maternity and Children Hospital (MMCH) The hospital is the main maternity and children referral hospital in the Madinah AI-Munawarah, .

The study investigated thirty children (2 -12 years.) who were proved to have steroid sensitive nephritic syndrome (SSNS) and, consecutively admitted in our department during the period from August 2010 to August 2012. Active stage of SSNS was defined as increased urinary protein excretion (Albustix ≥2+ for at least three consecutive days or > 40 mg/m^2 per hour) and serum albumin ≤25 g/l. Remission was defined as serum albumin concentration ≥ 35 g/l and normal protein excretion (Albustix trace or negative for at least three consecutive days or 5 mg/m^2 per hour) [23]

Children with SSNS were subdivided into 3 groups: group (A) Patients in the active stage (30 children), Patients in the remission stage were further subdivided into two groups:

(B1) remission phase still on steroid dose of prednisone of 40 mg/m^2 on alternate day regimen (30 children) and, (B2) remission phase off steroid treatment for at least 6 months (21/30 children). Patients (30/30) were tested both in active stage and at the time of testing no patient or control was taking any immunomodulating drug (i.e. cyclosporine A, cyclophosphamide, levamisole or MMF), to avoid their effects on the they have history of recent (within the previous 6 months) infection and/or inflammatory conditions, or abnormal urinary sediments (abnormal casts or crystalluria). The control group (group C) included thirty age and sex matched healthy children aged 2-/12 years (male, 15 female, 15) with (urinary protein/ creatinine ratio (</0.2) and, serum albumin level (4.1±/4.6 mg/dl).

Ethical considerations:

The study was approved by the Ethics Committees of Madinah Maternity and Children Hospital (MMCH). All parents of the patients and the controls were informed about the study and written informed consent was obtained

Methods:

A comprehensive clinical examination was done by one of the investigators or his assistant to all individuals who consented to participate in the study. The examination aimed to elicit signs and symptoms of the disease, presence or absence of complications and risk factors for relapse (upper respiratory tract infections, fever, common cold, vaccinations…..).

The following investigations were routinely performed in all children in the laboratory of Maternity and Children Hospital.

Blood: full blood count, urea; Creatinine; liver function test; ASOT; C3/C4; Varicella titers , urine culture and urinary protein/creatinine ratio, urinalysis including glucose, in all children before commencing steroids and, hepatitis B status to exclude secondary hepatitis.

Serum cytokine assay:

Blood samples were collected from all patients and controls in the test tubes without anticoagulant under sterile conditions. Serum was separated by centrifugation at 300*g* for 10 min, then divided in small aliquots and stored at −80 ^{o}C for future serum cytokine assessment.

Serum IFN-γ, IL-2, IL-4, IL-13 and IL-18 concentrations were measure by using quantitative colorimetric sandwich ELISA kits purchased from R&D China Co. Ltd., Shanghai. Following the manufacturer's instructions,, Each cytokine sample was run in duplicate and the mean cytokine concentration was calculated

Statistical analysis:

Statistical analysis was performed using Statistical Package for the Social Sciences for Windows (SPSS version 17). Values were expressed as median and ranges. The non-parametric Wilcoxon signed-rank test was used to compare differences between study groups with paired data. For non-paired data, statistical significance was analyzed by the Mann–Whitney U test. Spearman's coefficient of correlation (r) and Regression analysis model were e used to determine the correlations. $P < 0.05$ was considered to be statistically significant.

Results:

The current study investigated 30 children (17 males and 13 females) in the active stage of SSNS aged from 2 to 12 years (median = 3.52 years). The same group of children was re-investigated during remission phase still on steroid treatment; 21/30 was tested in remission phase off steroids for at least 6 months as well. All 30 patients who were studied in remission still on steroids were on the same dose of prednisone (40 mg/m2 on alternate day regimen).the control group consisted of 30 healthy siblings of the patients (15 males and 15 females) aged from 2 to 12 years (median = 4.12 years). There was no statistically significant difference between the group of children with nephritic syndrome and the control group in regard to age or sex (($p > 0.05$).

Table 1 summarizes the results of serum IL-2, IFN-γ, IL-4, IL-13 and IL-18 levels and p values in all study groups .There was no significant difference in IL-2 levels between nephrotic children of all disease stages and controls ($p > 0.05$). Children with active stage had significantly lower levels of IFN-γ when compared with the two remission phases (remission on steroids, remission off steroids ($p = 0.005$, $p = 0.001$, respectively) and controls ($p = 0.007$), While there was no significant difference between controls and each of the two groups of remission ($p > 0.05$).

To the opposite children with active stage of SSNS had significantly higher levels of IL-4 when compared with the two remission phases (remission on steroids, remission off steroids and $p < 0.0001$, $p < 0.0001$, respectively). In comparison to the controls patients in both remission groups showed significantly elevated IL-4 levels ($p < 0.0001$ and $p = 0.034$ respectively). Regarding, serum IL-13 levels, they were significantly higher in the active stage of SSNS compared with the two remission groups ($p < 0.0001$, $p < 0.0001$, respectively). IL-13 levels, still elevated during both remission phases, compared with the controls ($p < 0.0001$ and $p = 0.002$, respectively). Worth notice were also the findings regarding IL-18 levels .In the active stage of SSNS .IL-18 levels were significantly higher compared with the two remission groups, and controls ($p = 0.003$, $p = 0.001$, and $p<0.0001$ respectively), and IL-18 levels, even lower than in the active stage, remained significantly higher in both remission groups, compared with the controls ($p<0.0001$, $p<0.0001$, respectively).

As shown in Fig. 1a and 1b, using the linear regression analysis serum IL-18 and IL-4 levels were significantly correlated in nephrotic children in all disease stages ($r = 0.72$ and $p<0.0001$).

In children in active stage of SSNS, serum IL-18 and IL-4 levels, were also significantly correlated ($r = 0.73$ and $p = 0.001$) Fig. 1b.

Fig .2a illustrates the significant correlation between serum IL-18 and IL-13 levels in nephrotic children in all disease stages ($r = 0.82$ and $p < 0.0001$). In children in active stage of SSNS, serum IL-18 and IL-4 levels, were also significantly correlated ($r = 0.76$ and $p = 0.001$) Fig. 2b.

Table 1: Serum Levels, median and range values of IL-2, (IFN-γ), IL-4, IL-13 and IL-18 in patients with different stages of SSNS and in controls.

serum cytokine level	Active state Group (A) (n = 30)	Remission on steroids Group (B1) (n = 30)	Remission off steroids Group (B2) (n = 21)	Controls Group (C) (n = 30)
IL-2(pg/ml) Median Range	8.3 8- 13.5	98 7.9- 12.8	9.1 7.6 – 12.9	8.7 7.9 – 13.1
	[a] P > 0.05*		[a] P > 0.05	[a] P > 0.05
(IFN-γ) (pg/ml) Median Range	16.25 14.76 – 29.9	24.5 13.72 – 36.9	25.9 15.28 - 35.7	21.8 13.4 – 33.3
		[a] P = 0.005 [c] P > 0.05	[a] P = 0.001 [c] P > 0.05	[a] P = 0.007
IL-4 (pg/ml) Median Range	65 19.7 – 109.5	33.7 15.8 – 81.8	21.9 11.6 – 69.5	14.5 7.7 - 42.
		[a, c] p < 0.0001	[a] p < 0.0001 c P = 0.034	[a] p < 0.0001
IL-13 (pg/ml) Median Range	59.9 32.5 – 160.8	28.7 11.8 – 133.7	19.7 11.1 - 96.3	12.7 10.4 – 20.6
		[a, c] p < 0.0001	[a] p < 0.0001 [c] p = 0.002	[a] p < 0.0001
IL-18 (pg/ml) Median Range	1564 532.1 - 2716	1311 417 – 2245	765 321 - 1278	109 29 - 236
		[a] p = 0.003 [c] p < 0.0001	[a] p = 0.001 [c] p < 0.0001	[a] p < 0.0001

P < 0.05 was considered to be statistically

[a] p; comparing active stage with remission on steroids, remission off steroids ,and controls.

[c] p;, comparing controls with remission on steroids, and remission off steroids.

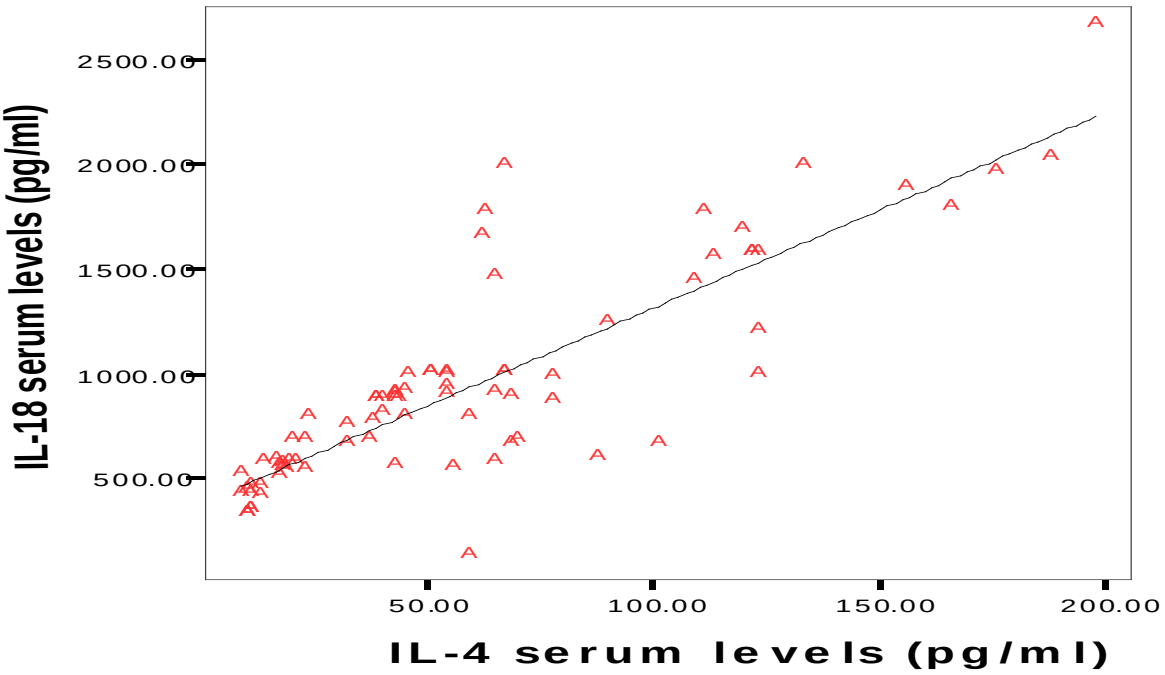

Figure 1a: Correlation of serum IL-18 and IL-4 levels in children with SSNS in all stages.

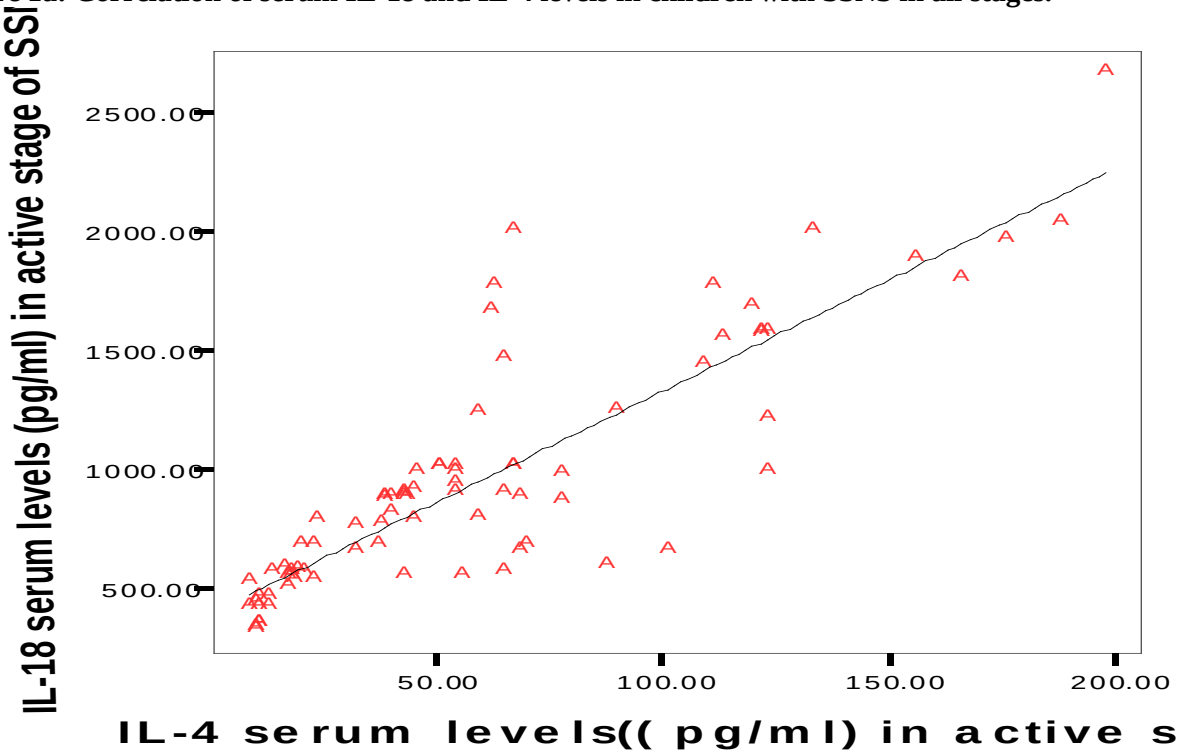

Figure 1b: Correlation of serum IL-18 and IL-4 levels in children with active stage of SSNS.

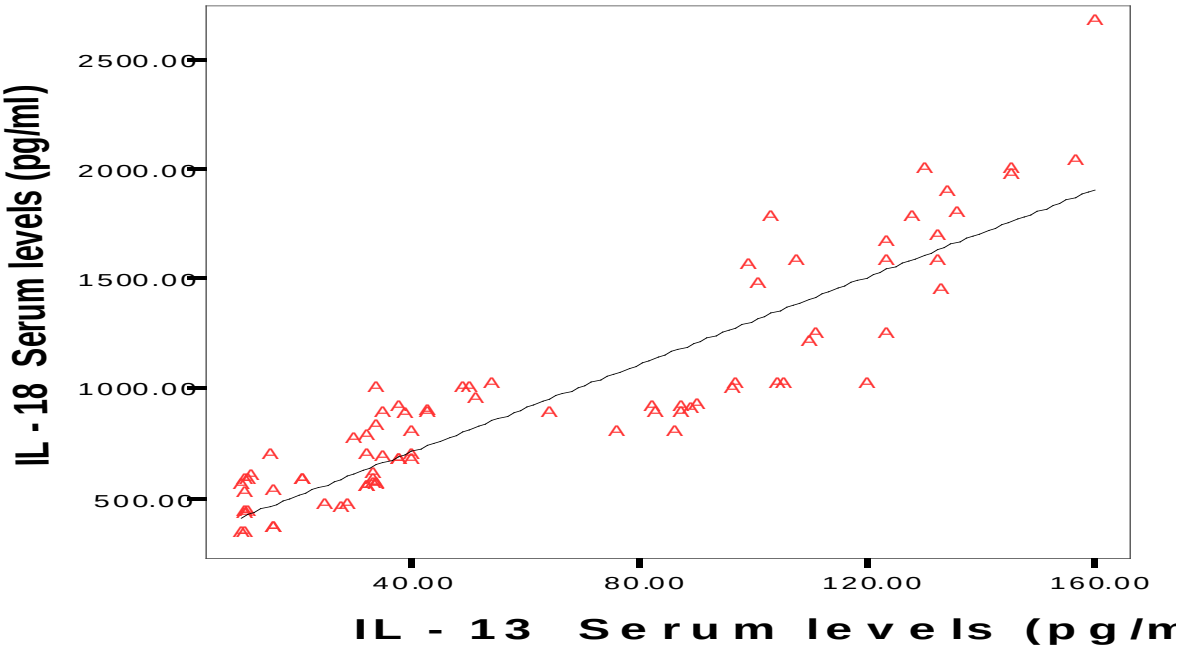

Figure 2a: Correlation of serum IL-18 and IL-13 levels in children with SSNS in all stages

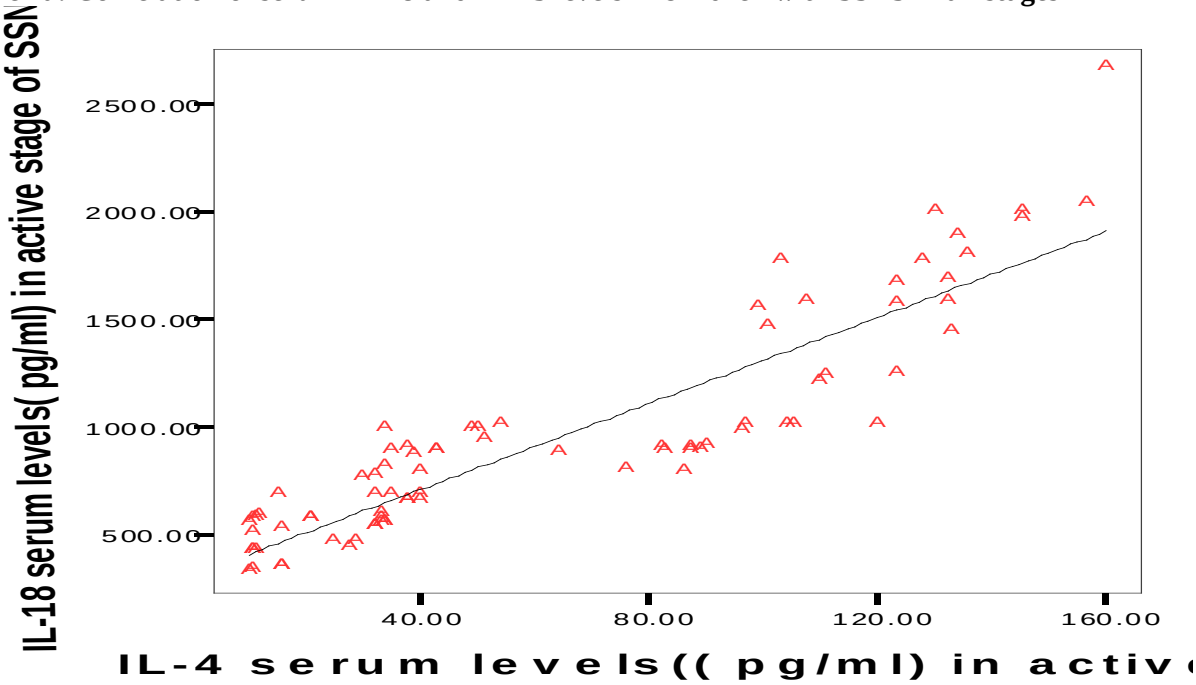

Figure 2b: Correlation of serum IL-18 and IL-13 levels in children with active stage of SSNS.

Discussion:

Studies of the type-1/type-2 cytokine patterns in the sera of patients with SSNS have generally been variable and inconsistent. This conflicting result may be due to the differences in immunologic techniques which were used to assess cytokine synthesis [24].

Our study has clearly shown that children with active SSNS showed a down-regulation of type-1 immune response. IL-2 serum levels were not significantly different in children with SSNS in all stages. This is in agreement with pervious cited studies [16–19].

Printza et al. [24] reported, in agreement with our findings, no significant difference of IL-2 serum levels in children with SSNS.

On the contrary Zachwieja et al. [16] demonstrated the presence of higher intracellular expression of IL-2 using a three-color flow cytometric assay. Considering the physiological fact that increased intracellular production does not usually results in increased secretion, we can partially explain the difference between our results and Zachwieja et al. [25].

In the group of children with active, IFN-γ serum levels were lower than that in both remission groups which were not significantly different from controls. Our results are in accordance with the results of two previous studies [24, 26] which showed a decreased production of IFN-γ by stimulated peripheral blood mononuclear cells in patients with active SSNS. Cheung et al. [27] and Kaneko et al. [28], who assessed the percentage of IFN-γ producing N cells in patients with SSNS, did not find significant difference between patients and controls. The predominance of type-2 immune response in our children with active SSNS is further supported by the Upregulation of the serum levels of IL-4. These results are in agreement with earlier published works by Stokowski et al. [20], Neuhaus et al. [9], and Cho et al. [10], who reported in patients with active SSNS an increased synthesis of IL-4 by stimulated peripheral mononuclear cells. Also the results of the current study are in agreement with Printza et al. [24] Kang et al. [29], and Lama et al. [30]. On the other hand, Cheung et al [27] did not report any increase in the percentage of IL-4 producing T cells.

Further support to the shift to type-2 immune response in our children with active SSNS is demonstrated by the increase of IL-13 serum levels in all our patients with SSNS. The results of this study are in accordance with pervious published studies [27, 19]. Yap et al. [19] demonstrated an elevated expression of IL-13 mRNA using a semi-quantitative reverse trancriptase PCR technique.

An experimental study on rat by Lai et al. [31] demonstrated the occurrence of podocyte injury, inducing a minimal-change like nephropathy with the over-expression of IL-13. Recently Printza et al. [24] reported that serum IL-13 levels were significantly higher in the active stage of SSNS compared with the two remission phases and that although IL-13 levels were even lower than in the active stage, they remained elevated during both remission phases, compared with the controls.

Our results highlight an important finding of increased levels of IL-18 in all disease stages, and particularly in the active stage of SSNS compared with the controls. This elevation should be greatly emphasized considering that IL-18 is a unique cytokine that can trigger both type-1 and type-2 immune responses depending on the predominant cytokines [2].

Earlier in the literature IL-18 was shown to be an IFN-γ trigger, which plays a critical role in the host defenses [32]. Recently, IL-18 has been postulated to induce IL-13 and/or IL-4 production by NK cells, mast cells and basophils [31, 32].

IL-18 is currently incriminated to play a potent role in various pathological conditions. The available data support its involvement in various diseases such as insulin dependent diabetes mellitus, rheumatoid arthritis, Chron's disease and atopy.

In collaboration with IL-12, IL-18 stimulates Th1-mediated immune responses, which play a critical role in the host defense against infection with intracellular microbes through the induction of IFN-gamma. However, the overproduction of IL-12 and IL-18 induces severe inflammatory disorders, suggesting that IL-18 is a potent proinflammatory cytokine that has pathophysiological roles in several inflammatory conditions [2]. IL-18 mRNA is expressed in a wide range of cells including Kupffer cells, macrophages, T cells, B cells, dendritic cells, osteoblasts, keratinocytes, astrocytes, and microglia. Thus, the pathophysiological role of IL-18 has been extensively tested in the organs that contain these cells. Somewhat surprisingly, IL-18 alone can stimulate Th2 cytokine production as well as allergic inflammation. Therefore, the functions of IL-18 in vivo are very heterogeneous and complicated. In principle, IL-18 enhances the IL-12-driven Th1 immune responses, but it can also stimulate Th2 immune responses in the absence of IL-12 [31].

Printza et al. [24] showed that in children with SSNS IL-18 levels were elevated in the active stage of the disease and that there was a positive correlation between IL-18 production and disease activity. Our results demonstrated that in SSNS, IL-18 was significantly correlated with I L-4 and IL-13 which are type-2 cytokines.

In conclusion, the current study reports that during the active stage of SSNS the balance is tipped in favor of type-2 cytokine pattern, and that apparently IL-18 is correlated with these type-2 cytokines.

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